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Attorney Docket No.: 100725-9

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

APPLICANTS : Florian Kern  
SERIAL NO. : 09/600,564  
FILED : November 7, 2000  
FOR : A Method for Identifying T-Cell  
Stimulating Protein Fragments  
ART UNIT : 1645  
EXAMINER : Zeman, Robert A.

**DECLARATION OF PROF. DR. DIRK BUSCH**

1. My name is Dirk Busch. I am a citizen of Germany residing at Liendlweg. 3, 81929 Munich, Germany.
2. My educational background is in the field of medical research (immunology, microbiology). I obtained the degree(s) of Medical Doctor from the University of Mainz.
3. I am head of a research unit at the Institute for Medical Microbiology, Immunology, and Hygiene, Technical University Munich, Trogerstr. 30, 81929 Munich. My unit is involved in the design, validation and standardization of antigen-specific flow-cytometry assays. My vitae is set forth in Exhibit I.
4. I am the principal author of more than X Medline indexed research articles in this field summarized in Exhibit II.
5. I have carefully studied the specification and claims in

the US patent application US 09/600564, and would like to make the following declaration.

6. The method described in the claims has become a household method in many research labs following its first publication in Nature Medicine in 1998 [Kern-F et al., T-cell epitope mapping by flow-cytometry, Nature Medicine, 1998, Aug;4(8):975-8 ]

7. The method is based on the short term stimulation of T lymphocytes with peptides where the T lymphocytes are contained in a cell suspension. Peptides are added for stimulation. In order to be able to stimulate the T-cells, the peptides need to be uploaded onto class-I Major Histocompatibility Complex (MHC) molecules, because this is the only way they can be recognized by T lymphocytes via the T cell receptor (TCR). Loading of the peptides onto the MHC may require shortening (clipping) of peptides by some as yet not precisely identified proteolytic mechanism. The loading of peptides, including the clipping, onto class-I MHC molecules is known to occur quite rapidly; typically, almost complete saturation will be achieved within the first 30 min of incubation at 37°C.

8. It is known that once T-cells are being stimulated, they start synthesizing molecules which can be used to identify such stimulation. The production of these molecules, among which are cytokines, follows different kinetics. 6 hours is known to be a time after which most cytokines can be found, in particular IFN-gamma, IL-2, and TNF. No one single time-point is optimum for all cytokines; however, such cytokines will have reached reach a point of maximum secretion at approximately 12 hours following stimulation.

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9. It is also known that, approximately 16 - 20 hours following stimulation, T lymphocytes may start replicating their DNA content in preparation for a cell division.

10. Typically, using the method referred to under 6., cell division will not occur within 24 hours after stimulation.

11. The description of the method in the claims of USSN 09/600564 states that

...the time of incubation [cell suspension plus peptides] should be sufficiently long so that the protein fragment or fragments are sufficiently taken up by the major histocompatibility antigen (MHC) molecules present on the cell surface, said taking up being sufficient when an unambiguous identification of stimulated T-cells is possible; and the incubation time of the suspensions containing T-cells with the protein fragment or fragments is sufficiently short so that selection and proliferation accompanied by the specific elimination of particular T-cells do not occur...


The specifications further teach that this incubation time can be 6 hours.

12. In light of my explanation of what is known to those skilled in the art, the description of the method in the specification of USSN 09/600564 gives sufficient guiding to anyone skilled in the art to perform the method claimed in USSN 09/600564. Specifically, setting up the assay with a 6 hour incubation time, and then working with longer and shorter incubation times will enable everybody to make use of the method and to find the optimum incubation period for their particular system.

13. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like are punishable by fine or imprisonment, or both, under 18 U.S.C 1001 and that such willful false statements may jeopardize the validity

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of the application or any patent issued thereon.

  
\_\_\_\_\_  
\_\_\_\_\_, Declarant

December 4<sup>th</sup> 2006  
\_\_\_\_\_  
Date

**Prof. Dr. med. Dirk Busch**  
Institut für Med. Mikrobiologie,  
Immunologie und Hygiene  
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## EXHIBIT I

## PERSONAL INFORMATION

Surname(s) / First name(s)

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E-mail(s), Web address(s)

Nationality(-ies)

Date of birth

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## WORK EXPERIENCE

• Dates (from – to)

Name and address of  
employer

Type of business or sector

Occupation or position held

Main activities and  
responsibilities

1994 – 1996

Children's Hospital, University of Würzburg, Germany

Intern and postdoctoral researcher at the Laboratory of Pediatric Rheumatology  
University of Würzburg

Intern and Postdoc

Patient care (30%), research (70%)

• Dates (from – to)

Name and address of  
employer

Type of business or sector

Occupation or position held

Main activities and  
responsibilities

1996 – 1999

Yale University, New Haven/USA

Postdoctoral researcher at the Section of Infectious Diseases and  
Immunobiology

Postdoc

research

• Dates (from – to)

Name and address of  
employer

Type of business or sector

Occupation or position held

Main activities and  
responsibilities

1999 – 2004

Institute for Medical Microbiology, Immunology and Hygiene  
Technical University Munich

medical research (immunology, microbiology)

Senior Researcher, group leader

Patient care (20%), research (80%)

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• Dates (from – to)	2004 - present
Name and address of employer	Institute for Medical Microbiology, Immunology and Hygiene Technical University Munich
Type of business or sector	medical research (immunology, microbiology)
Occupation or position held	principal investigator, C3 professor
Main activities and responsibilities	Patient care (20%), research (80%)

## EDUCATION

Date	1987 – 1993
Place of education	Mainz and Freiburg, Germany
Name and type of organisation providing education	University
Title or qualification awarded	Medicine (Staatsexamen)

Date	1991 – 1993
Place of education	Endocrinology (thesis work), Mainz, Germany
Name and type of organisation providing education	University of Mainz
Title or qualification awarded	Medical Doctor (M.D.)

Date	1994 – 1996
Place of education	University of Würzburg, Germany
Name and type of organisation providing education	Children's Hospital, University of Würzburg
Title or qualification awarded	Pediatrics

Date	1999 – present
Place of education	Munic, Germany
Name and type of organisation providing education	Institute for Medical Microbiology, Immunology and Hygiene Technical University Munich
Title or qualification awarded	Infectious Diseases (specialization)

## PERSONAL SKILLS AND COMPETENCIES

Mother tongue(s)	German
Other language(s)	
Language Speaking	English fluent

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Writing		good
Understanding (listening and reading)		good
Language		Spanish
Speaking		good
Writing		good
Understanding (listening and reading)		good
SOCIAL SKILLS AND COMPETENCIES		Group leader (laboratory) Family
ORGANISATIONAL SKILLS AND COMPETENCIES		Responsibilities for financial affairs and all employees at Institute for Medical Microbiology, Immunology and Hygiene, Technical University Munich (15 people)
TECHNICAL SKILLS AND COMPETENCIES		Basic techniques in molecular and cell biology, MHC multimers, (multi-parameter) flow cytometry, mouse genetics
ARTISTIC SKILLS AND COMPETENCIES		music (guitar)
OTHER SKILLS AND COMPETENCIES		
DRIVING LICENCE(S)		A, BE, C1E, ML
ADDITIONAL INFORMATION		
ANNEXES		

## EXHIBIT II

Publication List  
(2006)

(October 31,

## Research Articles

1. Huppertz H.I., S. Moesbauer, D.H. Busch, and Karch H. (1996) Lymphoproliferative responses to *Borrelia burgdorferi* in the diagnosis of Lyme arthritis in children and adolescents. *Eur J Pediatr.* 155:297-302.
2. Huppertz H.I., D.H. Busch, H. Schmidt, S. Aleksic, and Karch H. (1996) Diarrhea in young children associated with *Escherichia coli* non-O157 organisms that produce shiga-like toxin. *J. Pediatr.* 128:341-346.
3. Busch D.H., C. Jassoy, U. Brinckmann, H. Girschick, and Huppertz H.I. (1996) Detection of *Borrelia burgdorferi*-specific CD8+ cytotoxic T cells in patients with Lyme arthritis. *J. Immunol.* 157:3534-3541.
4. Busch D.H., and Huppertz H.I. (1997) A 13-year-old adolescent with Kawasaki disease presenting with massive unilateral cervical lymphadenopathy and polyarthritis. *Monatsschr. Kinderh.* 145:597-601.
5. Busch D.H., H.G.A. Bouwer, D. Hinrichs, and Pamer E.G. (1997) A nonamer peptide derived from the *Listeria monocytogenes* metalloprotease is presented to cytotoxic T lymphocytes. *Inf. Immun.* 65: 5326-5329.
6. Rutkowski S., D.H. Busch, and Huppertz H.I. (1997) Lymphocyte proliferation assay in response to *Borrelia burgdorferi* in patients with Lyme arthritis: analysis of Lymphocyte subsets. *Int. Rheumatol.* 17: 151-157.
7. Busch D.H., and Pamer E.G. (1998) MHC class I/peptide stability: implications for immunodominance, in-vitro proliferation, and diversity of responding CTL. *J. Immunol.* 160: 4441-4448.
8. Busch D.H., I.M. Pilip, S. Vijn, and Pamer E.G. (1998) Coordinate regulation of complex T cell populations responding to bacterial infection. *Immunity* 8: 353-362.
9. Busch D.H., I.M. Pilip, and Pamer E.G. (1998) Evolution of a complex T cell receptor repertoire during primary and recall bacterial infection. *J. Exp. Med.* 188: 61-70.
10. White D.W., A. McNeil, D.H. Busch, I.M. Phillip, E.G. Pamer and Harty J.T. (1999) Perforin-deficient CD8+ T cells priming and antigen-specific immunity against *Listeria monocytogenes*. *J. Immunol.* 162: 980-988.
11. Busch D.H. and Pamer E.G. (1999) T lymphocyte dynamics during *Listeria*



monocytogenes infection. *Immunol. Lett.* 65: 93-98.

12. Busch D.H., and Pamer E.G. (1999) Affinity maturation of complex epitope-specific T cell populations responding to bacterial infection. *J. Exp. Med.* 189: 701-710.

13. Kerksiek K.M., D.H. Busch, I. Philip, S.E. Allen, and Pamer E.G. (1999) H2-M3 restricted T cells: rapid effector function during primary bacterial infection and diminished memory response. *J. Exp. Med.* 190:195-204.

14. Hanke T., H. Takizawa, C.W. McMahon, D.H. Busch, E.G. Pamer, J.D. Altman, Y. Liu, D. Cado, F.A. Lemonnier P.J. Bjorkman, and Raulet D.H. (1999) Direct assessment of MHC class I binding by seven Ly49 inhibitory NK cell receptors. *Immunity* 11:67-77

15. Huppertz H.J., S. Rutkowski, D.H. Busch, R. Eisebit, R. Lissner, and Karch H. (1999) Bovine colostrum ameliorates diarrhea in infection with diarrheagenic *Escherichia coli*, shiga toxin-producing *E. coli*, and *E. coli* expressing intimin and hemolysin. *J. Pediatr Gastroenterol Nutr* 29:452-456

16. Busch D.H., K. M. Kerksiek, and Pamer E.G. (2000) Differing roles of inflammation and antigen in T cell proliferation and memory generation. *J. Immunol.* 164: 4063-4070.

17. Kerksiek K.M., D.H. Busch and Pamer E.G. (2000) Variable Immunodominance hierarchies for H2-M3 restricted N- formyl peptides following bacterial infection. *J Immunol* 166; 1132-1140.

18. Yajima T, Nishimura H, Ishimitsu R, Yamamura K, Watase T, Busch DH, Pamer EG, Kuwano H, Yoshikai Y. (2001) Memory phenotype CD8(+) T cells in IL-15 transgenic mice are involved in early protection against a primary infection with *Listeria monocytogenes*. *Eur J Immunol* 31:757-766.

19. Wolan DW, Teyton L, Rudolph MG, Villmow B, Bauer S, Busch DH\*, Wilson I.A. Crystal structure of the murine NK cell-activating receptor NKG2D at 1.95 Å (2001) *Nature Immunol.* 2; 248-254 (\* co-senior author)

20. Benz C., Utermöhlen O., Wulf A., Villmow B., Dries V., Goeser T., Koszinowski U., and Busch D.H. (2002). Activated virus-specific T cells are early indicators of anti-CMV immune reactions in liver transplant patients. *Gastroenterology*, 122; 1201-1215.

21. Knabel M, Franz TJ, Schiemann M, Wulf A, Villmow B, Schmidt B., Bernhard H., Wagner H, and Busch DH (2002). Reversible MHC multimer staining for functional isolation of T cell populations and effective adoptive transfer. *Nature Medicine*, 8; 631-637.

22. Krmpotic A., Busch D.H., Gebhardt F., Bubic I., Hengel H., Hasan M., Scalzo A.A., Koszinowski U.H. and Jonjic S. (2002). MCMV glycoprotein gp40 confers virus resistance to CD8+ T cells and NK cells *in vivo*. *Nature Immunology*, 3:529- 535.

23. Vuylsteke R.J.C.L.M., van Leeuwen P.A.M., Meijer S., Wijnands P.G.J.T.B., Statius

Muller M.G., Busch D.H., Scheper R.J. and de Gruijl T.D (2002). A novel method of sampling tumour draining lymph nodes for phenotypic and functional analysis of dendritic cells and T-cells. *Am J Pathology*, 161:19-26.

24. Prazeres da Costa C., Kirschning C.J., Busch D., Dürr S., Jennen L., Heinzmann U., Prebeck S., Wagner H. and Miethke T. (2002). Role of chlamydial heat shock protein 60 in the stimulation of innate immune cells by *Chlamydia pneumoniae*. *Eur J Immunol* 32: 2460-2470.

25. Benz C., Holz G., Michel D., Awerkwiew S., Dries V., Sippel D., Goeser T., and Busch D.H. (2003). Viral escape and T cell immunity during gancyclovir-treatment of CMV-infection after simultaneous pancreas-kidney transplantation. *Transplantation* 15;75(5):724-727.

26. Yajima T., Nishimura H., Ishimitsu R., Watase T., Busch D.H., Pamer E.G., Kuwano H., Yoshikai Y.(2002). Overexpression of IL-15 in vivo increases antigen-driven memory CD8+ T cells following a microbe exposure. *J Immunol* 168(3):1198-203.

27. Kwok L.Y., Lütjen S., Soltek S., Soldati D., Busch D.H., Deckert M., Schlüter D. (2003).The induction and kinetics of antigen-specific CD8 T cells are defined by the stage-specificity and compartmentalization of the antigen in murine toxoplasmosis. *J Immunol* 15;170(4):1949-57.

28. Drexler I., Staib C., Kastenmüller W., Stefanovic S., Schmidt B., Lemonnier F.A.G., Rammensee H.G., Busch D.H., Bernhard H., Erfle V., Sutter G. (2003). Identification of vaccinia virus epitope-specific HLA-A\*0201-restricted T cell responses and comparative analysis of immunogenicity and protective capacity of smallpox vaccines. *PNAS* 100(1):217-22

29. Kerksiek K.M., Ploss A., Leiner I., Busch D.H., Pamer E.G. (2003). H2-M3 restricted memory T cells: persistence and activation without expansion. *J Immunol* 15;170(4):1862-9.

30. Heit A., Maurer T., Hochrein H., Bauer S., Huster K.M., Busch D.H., Wagner H. (2003). Toll like receptor 9 (TLR9) expression is not required for CpG-DNA aided cross-presentation of DNA conjugated antigens, but essential for cross-priming of CD8 T-cells. *J Immunol* 15;170(6):2802-5.

31. Cosma A., Nagaraj R., Bühler S., Busch D.H., Sutter G., Goebel F.D., Erfle V. (2003) Therapeutic vaccination with recombinant modified vaccinia virus Ankara-HIV-1 nef elicits a strong Nef specific T-helper cell response in chronic HIV infected individuals. *Vaccine*, 8;22(1): 21-29.

32. Schiemann M., Busch V., Linkemann K., Huster K.M., and Busch D.H. (2003). Differences in maintenance of CD8+ and CD4+ bacteria-specific memory T cell populations. *Eur. J. Immunol.* 33(10): 2875-2885.

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- (2003). Vaccination with Plasmid DNA activates Dendritic cells via Toll like receptor 9 (TLR9) but functions in TLR9 deficient mice. *J. Immunol.* 171:5908-5912.
34. Fleischer K., Schmidt B., Kastenmüller W., Busch D.H., Drexler I., Sutter G., Heike M., Peschel C., and Bernhard H. (2004). Melanoma-reactive class I-restricted cytotoxic T cells are stimulated and expanded by dendritic cells loaded with synthetic peptides, but fail to respond to dendritic cells presenting melanoma-derived heat shock proteins. *J. Immunol.* 172(1):162-9.
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36. Heit H., Huster K.M., Schmitz F., Schiemann M., Busch D.H., and Wagner, H. (2004) CpG-DNA aided cross-priming by cross-presenting B cells. *J. Immunol.* 172: 1501-1507.
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38. Huster K.M., Busch V., Schiemann M., Linkermann K., Kerksiek K.M., Wagner H. and Busch D.H. (2004). Selective expression of IL-7 receptor on memory T cells identifies early CD40L-dependent generation of distinct CD8+ memory T cell subsets. *PNAS* 101(15):5610-5.
39. Da Costa C.U, Wantia N., Kirschning C.J., Busch D.H., Rodriguez N., Wagner H., Miethke T. (2004) Heat shock protein 60 from Chlamydia pneumoniae elicits an unusual set of inflammatory responses via Toll-like receptor 2 and 4 in vivo. *Eur. J. Immunol.* 34(10):2874.
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41. Kuon W., Kuhne M., Busch D.H., Atagunduz P., Seipel M., Wu P., Morawietz L., Fernahl G., Appel H., Weiss E.H., Krenn V., and Sieper J. (2004) Identification of Novel Human AggreCAN T Cell Epitopes in HLA-B27 Transgenic Mice Associated with Spondyloarthritis. *J. Immunol.* 15;173(8):4859-66.
42. Krmpotic A., Hasan M., Loewendorf A., Saulig T., Halenius A., Lenac T., Polic B., Bubic I., Kriegeskorte A., Messerle M., Hengel H., Busch D.H., Koszinowski U.H., Jonjic S. (2005) NK cell activation through the NKG2D ligand MULT-1 is selectively prevented by the glycoprotein encoded by mouse cytomegalovirus (MCMV) gene m145 product. *J Exp Med.* 17;201(2):211-20
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in vivo. *Blood* 15;105(2):742-9.

44. Bernhard H., Schmidt B., Busch D.H., and Peschel C. (2005) Isolation and expansion of tumor-reactive cytotoxic T cell clones for adoptive immunotherapy. *Methods Mol Med.* 2005;109:175-84

45. Adam C., King S., Allgeier T., Braumüller H., Mysliwicz J., Kriegeskorte A., Busch D.H., Röcken M., and Mocikat R. (2005) DC-NK cell cross-talk as a novel CD4+ T cell-independent pathway for antitumor CTL induction. *Blood* 106(1):338-344.

46. Heit A., Schmitz F., Staib C., O'Keeffe M., Busch D.H., Wagner H., and Huster K.M. (2005) Protective CD8 T Cell Immunity Triggered by CpG-Protein Conjugates competes with the efficacy of live vaccines. *J Immunol.* 174(7):4373-80.

47. Pasche B., Kalaydjiev S., Franz T. J., Kremmer E., Gailus-Durner V., Fuchs H., Hrabé de Angelis M., Lengeling A., Busch D.H. (2005) Sex dependent susceptibility pattern to *Listeria monocytogenes* infection is mediated by differential IL-10 production. *Infection and Immunity* 73(9):5952-60.

48. Semmrich M., Feterowski C., Beer S., Engelhardt B., Busch D.H., Bartsch B., Pfeffer K., Holzmann B. (2005) Critical importance of integrin LFA-1 deactivation for the generation of immune responses. *JEM* 201(12):1987-1998.

49. Kriegeskorte A.K., Gebhardt F.E., Porcelli S., Sternberger C., Schiemann M., Franz T., Huster K. M., Colonna M., Yokoyama W., Sicchardi A., Bauer S., and Busch D.H. (2005) Suppression of T cell proliferation by distinct NKG2D-ligands. *PNAS* 16;102(33):11805-10.

50. Pérez de Lema G., Maier H., Franz T., Escribese M., Chilla S., Camarasa N., Schmid H., Banas B., Segerer S., Kalaydjiev S., Busch D.H., Pfeffer K., Mampaso F., Schlöndorff D., Luckow B. (2005) Ccr2 deficiency reduces renal disease and prolongs survival in MRL/lpr lupus-prone mice. *J Am Soc Nephrol.* 16(12):3592-601.

51. Gailus-Durner V.\*, Fuchs H.\*, Brielmeier M., Calzada-Wack J., Elvert R., Ehrhardt N., Dalke C., Franz T.J., Grundner-Culemann E., Hammelbacher S., Hölter S.M., Horsch M., Javaheri A., Kalaydjiev S., Klempt M., Kunder S., Lengger C., Lisse T., Mijalski T., Naton B., Pedersen V., Prehn C., Przemeck G., Racz I., Reinhard C., Reitmair P., Schneider I., Steinkamp R., Zybille C., Adamski J., Beckers J., Behrendt H., Favor J., Graw J., Heldmaier G., Höfler H., Ivandic B., Katus H., Kirchhof P., Klingenspor M., Klopstock T., Lengeling A., Müller W., Ohl F., Ollert M., Quintanilla-Fend L., Schmidt J., Schulz H., Wolf E., Wurst W., Zimmer A., Busch D.H.\*, and Hrabé de Angelis M (2005) Introducing the German Mouse Clinic: Open access platform for standardized phenotyping. *Nature Methods* 2(6):403-4. (\*contributed equally)

52. Massberg S., Konrad I., Schürzinger K., Lorenz M., Schneider S., Zohlnhoefer D., Hoppe K., Schiemann M., Kennerknecht E., Sauer S., Rudelius M., Seidl S., Schulz C., Sorge F., Langer H., Peluso M., Goyal P., Vestweber D., Emambokus N.R., Busch D.H.

- Frampton J, and Gawaz M. (2006) Platelets secrete SDF-1 $\alpha$  and recruit bone marrow-derived progenitor cells to arterial thrombi in vivo. *J Exp Med*. 15;203(5):1221-33.
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54. del Barco Barrantes I., Montero-Pedrazuela A, Guadaño-Ferraz A., Obregon M.J., Martinez de Mena R., Gailus-Durner V., Fuchs H., Franz T., Kalaydjiev S., Klempt M., Hölter S., Rathkolb B., Reinhard C., Morreale de Escobar G., Bernal J., Busch D.H., Wurst W., Wolf E. Schulz H., Shtrom S., Wanker E., Hrabé de Angelis M., Westphal H., and Christof Niehrs C. (2006) Generation and characterization of *dickkopf3* mutant mice. *Mol Cell Biol* 26(6):2317-26.
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61. Neudorfer J., Schmidt B., Huster K., Anderl F., Schiemann M., Schmidt T., Wagner H., Peschel C., Busch D.H. and Bernhard H. (2005) Reversible HLA multimers (streptamers)

for isolation of human cytotoxic T lymphocytes functionally active against tumor- and virus-derived antigens. *submitted for publication (Cancer Research)*

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